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Effect of asymmetric terminal structures of short RNA duplexes on the RNA interference activity and strand selection

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ABSTRACT

Short interfering RNAs (siRNAs) are valuable reagents for sequence-specific inhibition of gene expression via the RNA interference (RNAi) pathway. Although it has been proposed that the relative thermodynamic stability at the 5′-ends of siRNAs plays a crucial role in siRNA strand selection, we demonstrate here that a character of the 2-nt 3′-overhang of siRNAs is the predominant determinant of which strand participates in the RNAi pathway. We show that siRNAs with a unilateral 2-nt 3′-overhang on the antisense strand are more effective than siRNAs with 3′-overhangs at both ends, due to preferential loading of the antisense strand into the RNA-induced silencing complex (RISC). Regardless of the relative thermodynamic stabilities at the ends of siRNAs, overhang-containing strands are predominantly selected as the guide strand; whereas, relative stability markedly influences opposite strand selection. Moreover, we show that sense strand modifications, such as deletions or DNA substitutions, of siRNAs with unilateral overhang on the antisense strand have no negative effect on the antisense strand selection, but may improve RNAi potency. Our findings provide useful guidelines for the design of potent siRNAs and contribute to understanding the crucial factors in determining strand selection in mammalian cells.

INTRODUCTION

RNA interference (RNAi) is a conserved regulatory mechanism of posttranscriptional gene silencing by which double-stranded RNA (dsRNA) induces sequence-specific degradation of homologous mRNA (1,2). RNAi was first discovered in *Caenorhabditis elegans* and has become a powerful technique in functional analyses of genes of interest. Although longer dsRNAs induce toxicity due to an interferon (IFN) response in mammalian cells, short interfering RNA (siRNA) can circumvent the IFN response (3), allowing siRNAs to be used in therapeutic applications. siRNAs consist of two complementary strands of ~21-nt with 2-nt overhangs at both 3′-ends (4). One strand of the duplex is selected as the 'guide strand' during assembly into the RNA-induced silencing complex (RISC) and directs this complex to the complementary mRNA target (5). Argonaute 2 (Ago2), the catalytic component of the RISC, catalyzes the cleavage of target mRNA between the 10th and 11th nucleotides measured from the 5′-end of the guide strand (4,6,7).

There is great interest in clarifying the sequence and structural requirements of siRNAs to improve their efficacy. Systematic studies using *Drosophila* embryo lysates revealed that duplexes with 21-nt strands and 2-nt 3′-terminal overhangs were more effective than those with longer strands (up to 25 nt) or shorter strands (20 nt) (4). Phosphorylation of the 5′-hydroxyl terminus of the antisense strand is essential for RNAi activity, whereas the 3′-hydroxyl terminus has little effect on the activity (8–10). Internal chemical modifications including 2′-*O*-methyl modification

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or locked nucleic acids (LNA) modification at specific positions are tolerated without significant loss of the RNAi activity and improve the stability of siRNAs (9,11,12). Reynolds *et al.* (13) determined internal properties of siRNA duplexes that influence siRNA function through the analyses of 180 siRNAs, leading to an algorithm to design potent siRNAs. Several studies based on computational modeling and experimental data demonstrated that the secondary structure of targets and accessibility to them were also important for determining siRNA activities (14–17).

Selective loading of the antisense strand into the RISC is essential for avoiding undesirable side effects. In RNAi, strand selection is biased, and the relative stabilities of the base pairs at the 5′-ends of the two siRNA strands were proposed to determine the strand selection for siRNAs as well as microRNA (miRNA) duplexes (18,19). In *Drosophila*, a heterodimer with *Drosophila* dicer (Dcr-2) and dsRNA-binding partner R2D2 determines asymmetric loading of siRNA strands into the RISC (20). R2D2 binds to the 5′-end with a greater internal stability and directs the Dcr-2 to near the 5′-end of the opposite strand to be loaded into the RISC. In mammalian cells, co-factor(s) interacting with dicer may play an important role in the strand selection as in the fruit fly, but the details have not been established.

Recent studies gave rise to the question of whether structural properties of siRNAs are involved in the strand selection in mammalian cells. Vermeulen *et al.* (21) reported that siRNAs with unilateral 2-nt 3′-overhang on the antisense strand were more effective than those with 3′-overhangs or unilateral 2-nt 3′-overhang on the sense strand. Another group also showed potent activity of siRNAs with unilateral overhangs termed 'fork-siRNA duplexes' compared with siRNAs with overhangs (22). Although these studies suggest that terminal structures of siRNA might be involved in strand selection, further details remain unexamined. Here, we demonstrate that siRNAs with unilateral 2-nt 3′-overhangs on the antisense strand [asymmetric 2-nt antisense strand overhang (o-b); Figure 1A] are more potent than siRNAs with symmetric overhangs [symmetric 2-nt overhangs (o-o); Figure 1A], due to preferential antisense strand selection. We also examined the effect of other factors, including relative thermodynamic stability and strand modifications, and found that the terminal structure of siRNA is the primary factor affecting the strand selection, as well as the RNAi activity. Our findings provide technical guidelines for the design of siRNAs with enhanced potency and help in the understanding of the crucial factors in determining strand selection in mammalian cells.

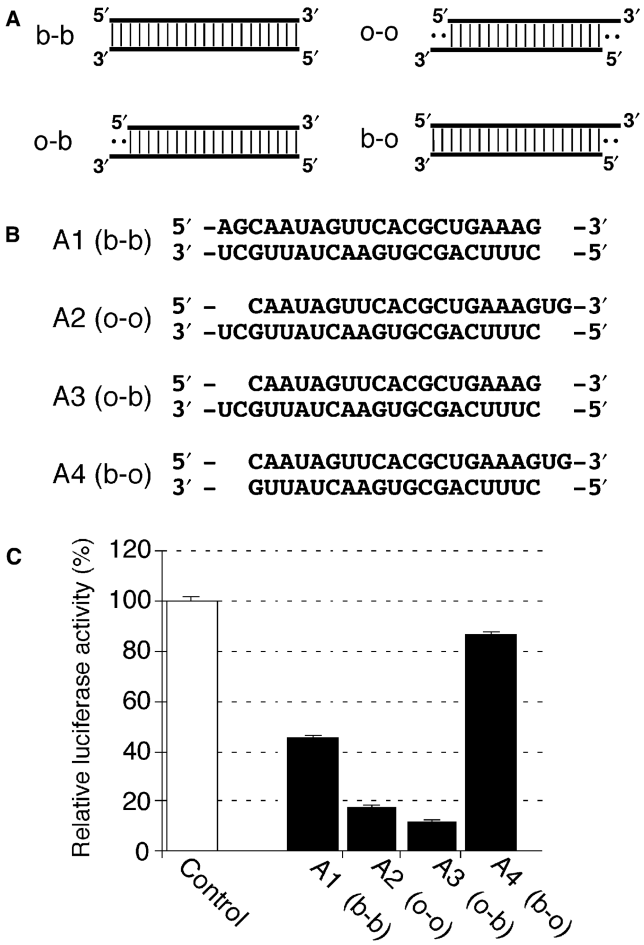

Figure 1. Structures and activities of siRNA duplexes. (**A**) Schematic representation of siRNA duplexes with symmetric or asymmetric overhangs. Each duplex is indicated as b-b: symmetric blunt ends; o-o: symmetric 2-nt 3′-overhangs; o-b: asymmetric antisense strand overhang; and b-o: asymmetric sense strand overhang. (**B**) Sequences of duplexes with symmetric or asymmetric overhangs targeted against the gene for *Renilla* luciferase. (**C**) Inhibition of the reporter gene expression by various siRNA duplexes. The *Renilla* luciferase expression plasmid, firefly luciferase expression plasmid and 0.05-nM siRNA were cotransfected into HeLa S3 cells, and luciferase activities were determined. The luciferase activities determined from cells transfected with the control siRNA were set at 100%. The mean and SD from three replicate experiments are presented.

MATERIALS AND METHODS

Synthetic siRNAs

RNAs were designed and provided by iGENE Therapeutics Inc. (Tokyo, Japan). siRNA duplexes were prepared by mixing equimolar amounts of sense and antisense RNAs in an annealing buffer and heating at 96°C for 2 min, followed by slow cooling to room temperature (70–25°C) over 1 h.

Plasmid construction

To create reporter plasmids pGL3-RL-Fwd and pGL3-RL-Rev, a fragment of the *Renilla* luciferase coding region (372–487 nt position) was amplified by polymerase chain reaction (PCR) from the pRL-TK plasmid (Promega, Madison, WI, USA) using primers containing an *Xba* I restriction site and the *Renilla* luciferase sequence, as follows: sense (5′-AAAAAATCTAGATTTGGCATTTCAT TATAGC-3′) and antisense (5′-AAAAAATCTAGATAT CTTCTTCAATATCAGG-3′). PCR products were digested with *Xba* I and cloned into the *Xba* I site of the pGL3 plasmid (Promega) in both orientations. To create

psiCHECK-RL-Fwd or psiCHECK-RL-Rev, the hybridized sense (5′-TCGAAGCAATAGTTCACGCTGAAAGTG-3′ or 5′-TCGACACTTTCAGCGTGAACTATTGCT-3′) and antisense (5′-GGCCCACTTTCAGCGTGAACTATTGCT-3′ or 5′-GGCCAGCAATAGTTCACGCTGAAAGTG-3′) strands, which corresponds to the *Renilla* luciferase coding region (414–436 nt position) were inserted into *Xho* I and *Not* I sites of the psiCHECK-2 plasmid (Promega), respectively. To create psiCHECK-RL2-Fwd or psiCHECK-RL2-Rev, the hybridized sense (5′-TCGAATCAAAGCAATAGTTCACGCTGAAAGTG-3′ or 5′-TCGACACTTTCAGCGTGAACTATTGCTTTTGAT-3′) and antisense (5′-GGCCCACTTTCAGCGTGAACTATTGCTTTGAT-3′ or 5′-GGCCATCAAAGCAATAGTTCACGCTGAAAGTG-3′) strands, which corresponds to the *Renilla* luciferase coding region (409–436 nt position) were inserted into the psiCHECK-2 plasmid (Promega), respectively.

Cell culture

HeLa S3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Co. St Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS; GIBCO/Invitrogen, Carlsbad, CA, USA) and antibiotics.

Transfection and luciferase assays

HeLa S3 cells were grown to ~70–80% confluency in 24-well plates and co-transfected with 100 ng of the firefly luciferase expression plasmid pGL3, 20 ng of *Renilla* luciferase expression plasmid pRL-RSV and 0.05–10 nM of siRNA. siRNA targeted against a gene for enhanced green fluorescent protein was used as a negative control. Transfection was performed with Lipofectamine^{TM} 2000 reagent (Invitrogen) according to the manufacturer's instructions. Activities of firefly and *Renilla* luciferases were analyzed with the Dual-Luciferase Reporter Assay System (Promega) 24 h after transfection. For transfection with pGL3-RL-Fwd or pGL3-RL-Rev, 100 ng of each reporter plasmid, 50 ng of pSV-β-galactosidase control vector (Promega) were cotransfected with the indicated amounts of siRNA. Twenty-four hours after transfection, cells were lysed with the reporter lysis buffer. Luciferase and β-galactosidase activities were analyzed with the Luciferase Assay System (Promega) and the β-Gal Reporter Gene Assay (Roche, Nutley, NJ, USA) according to the manufacturer's instructions, respectively. For transfection with psiCHECK-RL-Fwd or psiCHECK-RL-Rev, HeLa S3 cells were grown to ~70–80% confluency in 48-well plates and cotransfected with 35 ng of each luciferase reporter plasmid and 1 nM of siRNA. Luciferase activities were analyzed with the Dual-Luciferase Reporter Assay System (Promega) 24 h after transfection.

Northern blot analysis

Total RNAs were extracted and purified with ISOGEN^{TM} reagent (Wako, Osaka, Japan) according to the manufacturer's instructions. Twenty micrograms of total RNA per lane were loaded in a 10% polyacrylamide denaturing gel. Following electrophoresis, bands of RNA were electro-transferred to a Hybond-N^{+} membrane (Amersham Bioscience, Little Chalfont, UK). The membrane was probed with γ-^{32}P-labeled synthetic oligonucleotides complementary to the sense or antisense sequences of siRNAs, as follows: S probe (for A2, A3 and A4), 5′-CTTTCAGCGTGAACTATTG-3′; AS probe (for A2, A3 and A4), 5′-CAATAGTTCACGCTGAAAG; S probe (for RL-a1, RL-a2 and RL-a3), 5′-CTTATCTTGATGCTCATAG-3′; AS probe (for RL-a1, RL-a2 and RL-a3), 5′-CTATGAGCATCAAGATAAG-3′;.

Western blot analysis

HeLa S3 cells were transfected with 10- or 50-nM siRNAs targeted against *GAPDH* or *Lamin A/C*. Sixty hours after transfection, cells were lysed with lysis buffer [50 mM Tris–HCl, pH 8.0, 0.15 M NaCl, 1 mM EDTA, 1% NP40 and a complete protease inhibitor (Roche)] and incubated for 15 min on ice. Protein samples were separated by 10% SDS–PAGE and electro-transferred to a Hybond-P membrane (Amersham Biosciences). Membrane was blocked with the 5% ECL Blocking Agent (Amersham Biosciences) and then incubated with primary antibodies: monoclonal anti-Lamin A/C (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit polyclonal anti-GAPDH antibodies overnight at 4°C. The membrane was washed and incubated with secondary antibodies: horseradish peroxidase-linked anti-mouse or anti-rabbit antibodies (Amersham Biosciences) for 1 h at room temperature. Bands were visualized with the enhanced chemiluminescence system (ECL), according to the manufacturer's instructions (Amersham Biosciences).

RESULTS

Effect of terminal structures of siRNA duplexes on the RNAi activity

To investigate the effect of the terminal structures of siRNAs on the RNAi activity, we initially designed four types of siRNAs targeted against the gene for *Renilla* luciferase. These siRNA variants have different 3′-end structures: (i) symmetric blunt ends (b-b), (ii) symmetric 2-nt overhangs (o-o), (iii) asymmetric 2-nt antisense strand overhang (o-b) or (iv) asymmetric 2-nt sense strand overhang (b-o) (Figure 1A). Duplexes A1 and A2 consist of 21-mer sense and antisense strands, but A3 and A4 have 19-mer and 21-mer of strands (Figure 1B). To examine the RNAi activity of these siRNA variants, we cotransfected 0.05 nM of each siRNA with a plasmid encoding the *Renilla* luciferase gene and a plasmid encoding the firefly luciferase gene, into HeLa S3 cells. The ratio of *Renilla* luciferase versus firefly luciferase activities were determined 24 h after transfection. Although all duplexes showed a silencing effect against *Renilla* luciferase expression, we observed significant differences in activities of each type of siRNA (Figure 1C). A2 (o-o) was more active than A1 (b–b). Interestingly, A3 (o-b) showed greater activity than A2 (o-o); whereas, A4 (b-o) led to serious reduction of RNAi activity (Figure 1C). The same trend was observed in their activities using 0.1- and 0.5-nM siRNAs (Supplementary Figure 1).

Next, we examined the effect of siRNAs with distinct terminal structures on the expression of endogenous genes. Three types of siRNAs (o-o, o-b or b-o) targeted against either *Lamin A/C* or *GAPDH* were transfected into HeLa S3 cells and levels of expression of each protein were analyzed by western blot analysis (Figure 2A and B). The results obtained correlated well with the luciferase assay data shown in Figure 1C. siRNAs with an asymmetric antisense strand overhang (o-b) were the most effective, whereas siRNAs with an asymmetric sense strand overhang (b-o) showed less reduction in gene expression. For instance, siRNA (o-b) targeted against *GAPDH* reduced target expression by 80% compared with mock control while siRNAs (o-o) and (b-o) did so by 70% and 30%, respectively, using 50 nM of siRNA (Figure 2A). These results suggest that, in general, an asymmetric 2-nt overhang at the 3′-end of the antisense strand improves the RNAi potency. Sequences of siRNAs targeted against *Lamin A/C* and *GAPDH* are shown in Table S1 (Supplementary Data).

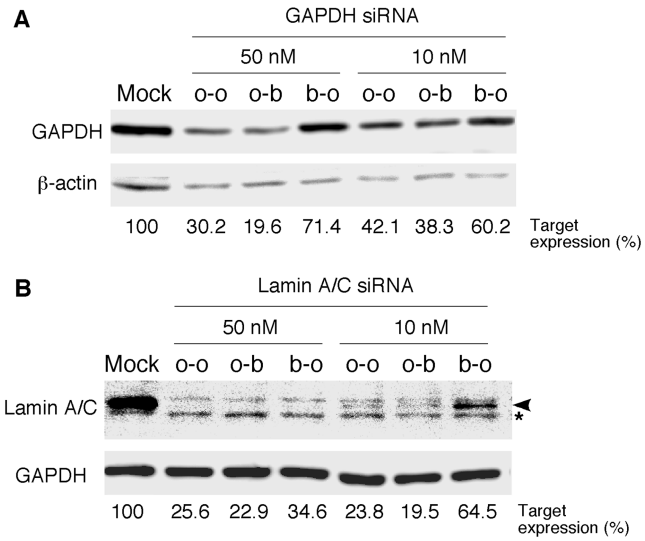

Figure 2. Effects of various siRNA duplexes on the expression of endogenous genes. (**A**) Inhibition of the GAPDH expression by siRNAs. HeLa S3 cells were transfected with 10- or 50-nM siRNAs targeted against *GAPDH*. Sixty hours after transfection, levels of GAPDH protein were determined by western blot analysis. Levels of β-actin protein were determined as an internal control. Expression levels of GAPDH normalized to those of β-actin in cells transfected with each siRNA are indicated. (**B**) Inhibition of the Lamin A/C expression by siRNAs. HeLa S3 cells were transfected with 10- or 50-nM siRNAs targeted against the *Lamin A/C* and levels of Lamin A/C protein were determined by the same procedure as (A). Levels of GAPDH protein were determined as an internal control. Bands that correspond to Lamin A/C are indicated by an arrowhead. An asterisk indicates nonspecific bands.

Terminal structures of siRNAs influence strand selectivity

Previous studies demonstrated that the strand selection of siRNAs depends on the thermodynamic stabilities of their 5′-ends (18,19). However, the terminal structure of siRNAs might be an important determinant of which strand participates in the RNAi pathway when relative thermodynamic stabilities at the 5′-ends of the siRNA are nearly equal. To examine whether the siRNA terminal structures influence the strand selectivity, we constructed reporter vectors harboring a 116-bp *Renilla* luciferase sequence in both orientations (forward or reverse) within the 3′-UTR of the firefly luciferase gene of the pGL3 plasmid, creating the pGL3-RL-Fwd (antisense complementary) and pGL3-RL-Rev (sense complementary) constructs (Figure 3A). These vectors readily allow the measure of relative incorporation of each siRNA strand into the RISC. HeLa S3 cells were cotransfected with the reporter vector, various siRNAs and a β-galactosidase expressing vector, which was used as a transfection control. Firefly luciferase versus β-galactosidase activities were analyzed 24 h after transfection (Figure 3B). Duplexes A1 (b-b) and A2 (o-o) efficiently inhibited the expression of firefly luciferase from both Fwd and Rev plasmids, indicating that both strands (sense and antisense) equally participate in the RNAi pathway. In contrast, the duplex A3 (o-b) showed strong suppression against the firefly luciferase sequence from pGL3-RL-Fwd, but was less active against the luciferase sequence from pGL3-RL-Rev. siRNA A4 (b-o) showed the opposite effects against each target. These results clearly indicate that the strand with the 2-nt overhang is preferentially incorporated into the RISC as the guide strand.

To investigate whether siRNAs with an asymmetric overhang generally show the expected strand selectivity against different target sites, we additionally synthesized siRNAs with distinct terminal structures against different regions of the *Renilla* luciferase gene and examined their activities in the luciferase assays (Table S1 and Figure 3C). As expected, all siRNAs with an asymmetric antisense overhang (o-b) showed strong suppression against the Fwd targets, and siRNAs with the opposite terminal structures (b-o) were effective against the Rev targets. Importantly, the RL-b2 (o-b) was more active against the Fwd targets than the conventional siRNAs RL-b1 (o-o). These results suggest that, in general, terminal structures play important roles in determining which strand participates in the RNAi pathway and asymmetric overhang structures possibly enhance the RNAi activity.

We next examined whether the sense or antisense strand of siRNAs with an asymmetric overhang selectively accumulates in cells using northern blot analyses. HeLa S3 cells were transfected with three types of siRNA [RL-a1 (o-o), RL-a2 (o-b) or RL-a3 (b-o); Table S1 and Figure 3C] and total RNAs were isolated 24 h after transfection. The RNAs were fractionated by electrophoresis and transferred to nylon membrane for probing with ^{32}P-labeled oligonucleotides complementary to either the sense strand (S probe) or antisense strand (AS probe) of the siRNAs. As shown in Figure 4A, both probes yielded signals with equal intensities in cells transfected with siRNA RL-a1 (o-o). In contrast, probes complementary to the sense strand of siRNA RL-a2 (o-b) and antisense strand of siRNA RL-a3 (b-o) yielded reduced signals compared with probes complementary to their opposite strand, respectively. These results suggest that strands with 3′ 2-nt overhang preferentially accumulate in cells relative to opposite strands when siRNAs with an asymmetric overhang were transfected into cells. However, we examined levels of other siRNAs (A2, A3 or A4; Figure 1B) and

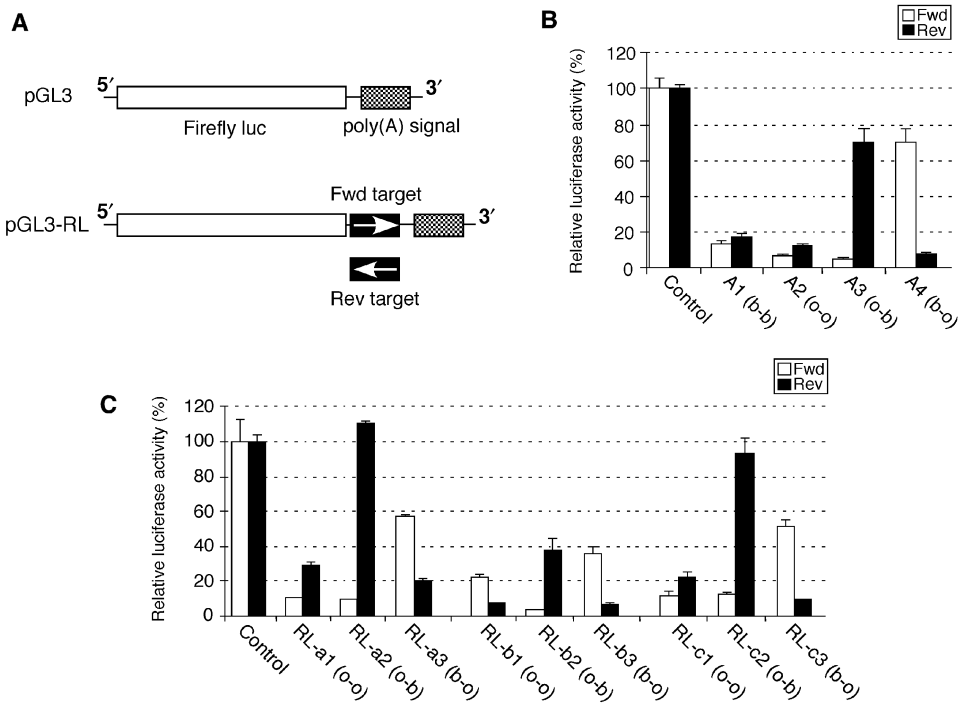

Figure 3. Effects of various siRNAs on the RNAi activity and strand selectivity. (**A**) Design of reporter constructs derived from plasmid pGL3. *Renilla* luciferase sequence was inserted into the 3′-UTR of firefly luciferase gene in the forward (Fwd) and in the reverse (Rev) orientation. (**B**) Inhibition of reporter gene expression by various siRNAs. Each of reporter constructs with a target sequence in the forward (Fwd) or the reverse (Rev) orientation, β-galactosidase expression plasmid and 0.5-nM duplexes were cotransfected into HeLa S3 cells, and luciferase and β-galactosidase activities were determined. The activities determined from cells transfected with the control siRNA were set at 100%. The mean and SD from three replicate experiments are presented. (**C**) Inhibition of reporter gene expression by various siRNAs targeted against different regions of the *Renilla* luciferase gene. The experimental procedure and values are the same as for (B), except that a concentration of duplexes is 10 nM.

obtained somewhat different results. Signals that corresponded to the sense strand of siRNA A3 (o-b) were markedly reduced compared with signals that corresponded to its antisense strand (Figure 4B), but unexpectedly, we observed no significant difference between signals that corresponded to sense and antisense strands of siRNA A4 (b-o). To test whether siRNA A4 (b-o) shows selective gene-silencing under these conditions, we cotransfected HeLa S3 cells with each siRNA and the reporter plasmid harboring the forward or the reverse target sequence, and luciferase activities were determined 24 h after transfection. As expected, siRNA A4 showed strong suppression of luciferase expression from the Rev plasmid, whereas it showed reduced activity against the Fwd target (Supplementary Figure 2C and D), indicating that the sense strand of siRNA A4 predominantly contributes the gene-silencing. We performed northern blotting in the presence of the target plasmid and obtained the same results as that obtained in Figure 4B (Supplementary Figure 2A and B). These results suggest that selective RNAi activity by siRNAs with an asymmetric overhang is not always a consequence of selective accumulation of their sense or antisense strands in cells.

Terminal structures of siRNAs are predominant determinants of strand selection

Although our results suggest that the terminal structures of siRNAs mainly determine which strand participates in the RNAi pathway, the extent of strand selectivity varied depending on siRNA sequences (Table S1 and Figure 3C). For instance, the siRNA RL-b2 (o-b) inhibited the Rev target expression to ~40% although the RL-a2 (o-b) had no effect on its expression (Figure 3C). This fluctuation seems to be associated with differences of relative thermodynamic stabilities at the 5′-ends of asymmetric siRNAs. To examine the relationship between terminal structure and 5′-end stability, the RNAi activity and strand selectivity of various siRNAs with asymmetric antisense strand overhangs were compared. We designed five siRNAs whose sequences were shifted in increments of 1 nt to the upstream side of the siRNA A3 (Figure 5A). These variants have different thermodynamic stabilities at their 5′-ends (Figure 5A). As shown in Figure 5B, the extent of sense-strand selectivity was influenced by relative thermodynamic stabilities. For instance, siRNA A3-1, which has lower internal stability at the 5′-end of the antisense strand, showed almost no activity against the Rev target. In contrast, siRNA A3-4, with a higher stability at the 5′-end of the antisense strand, showed strong activity against the Rev target. Surprisingly, however, all siRNA variants showed effective inhibition of the Fwd target expression regardless of thermodynamic stabilities at both ends.

To further examine whether terminal structure or relative thermodynamic stability is the more important determinant of strand selection, we synthesized siRNAs with a

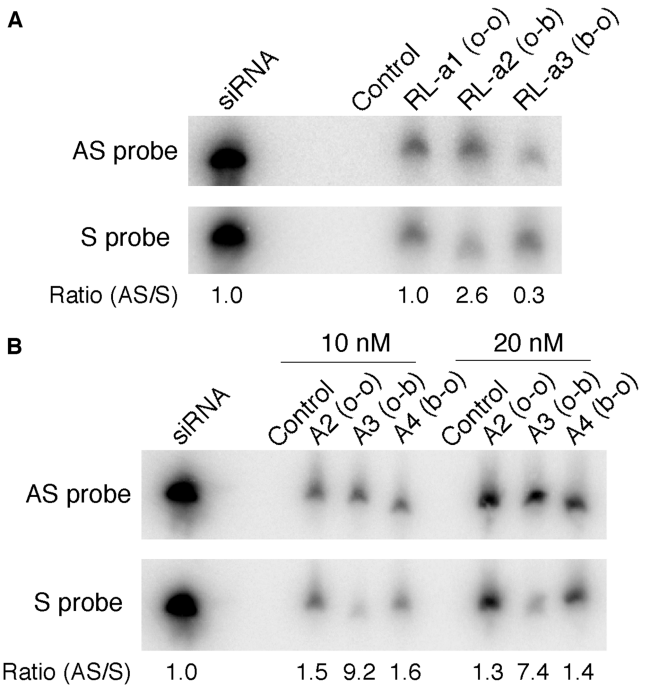

Figure 4. Northern blot analyses of various types of siRNAs. (**A**) Detection of siRNAs. Total RNAs were extracted from cells transfected with 10 nM of various types of siRNAs (RL-a1, RL-a2 or RL-a3) in a 6-well plate and northern blot analyses were performed with the specific probe complementary to the antisense strand (AS probe). The membrane was subsequently stripped and probed for the sense strand (S probe). Synthetic siRNA RL-a1 was loaded in a gel and used as a positive control (lane: siRNA). RNA prepared from cells transfected with an irrelevant siRNA was used as a negative control (lane: Control). The ratio of intensities of bands corresponding to antisense versus sense strands of the positive control was set at 1.0 and the ratios (AS/S) of each siRNA are indicated. (**B**) Northern blot analyses were performed as described as (A), except that 10- or 20-nM siRNAs (A2, A3 or A4) were used. Synthetic siRNA A2 was used as a positive control (lane: siRNA).

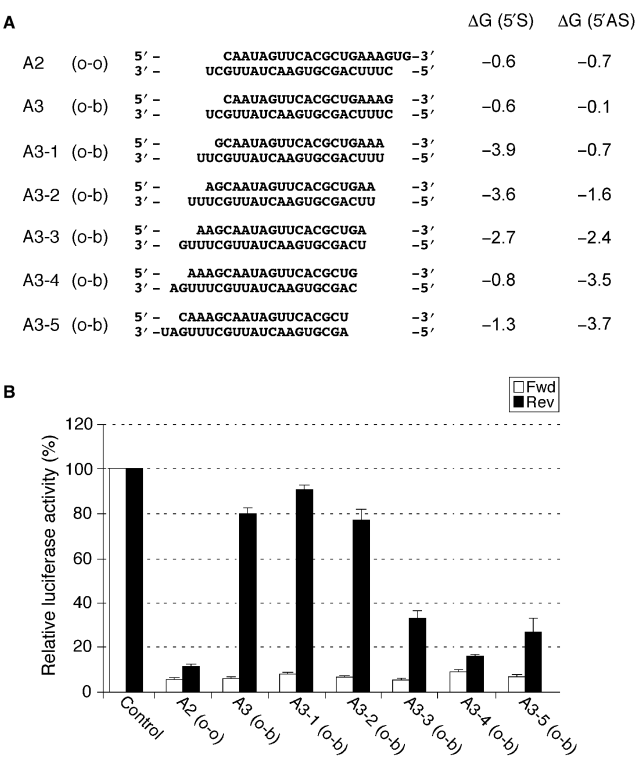

Figure 5. Effects of relative 5′-end stabilities on strand selectivity. (**A**) Sequences of various siRNAs with an asymmetric antisense strand overhang. Each siRNA sequence was shifted in increments of 1 nt to the upstream side of the siRNA A3. Predicted free energies (kcal/mol) of both ends (5′S; 5′-sense, 5′AS; 5′-antisense) of siRNAs are also indicated. (**B**) Inhibition of reporter gene expression by the series of siRNAs. Each of reporter constructs with a target sequence in the forward (Fwd) or the reverse (Rev) orientation, β-galactosidase expression plasmid and each 10-nM duplex were cotransfected into HeLa S3 cells, and luciferase and β-galactosidase activities were determined. The activities determined from cells transfected with the control siRNA were set at 100%. The mean and SD from three replicate experiments are presented.

G:U wobble pair or a mismatch at either end (Figure 6A). These mutations can shift the balance between relative stabilities at the ends of the siRNAs. We constructed reporter vectors harboring a short-target sequence in two orientations (forward or reverse) within the 3′-UTR of the *Renilla* luciferase gene of the psiCHECK-2 plasmid, creating the psiCHECK-RL-Fwd or psiCHECK-RL-Rev constructs. siRNAs that we used here did not interfere with the expression of *Renilla* luciferase gene from the psiCHECK-2 plasmid because this plasmid encodes a modified *Renilla* luciferase gene, which does not contain the target sequence for our siRNAs. HeLa S3 cells were cotransfected with the reporter vector and various siRNAs, and *Renilla* luciferase versus firefly luciferase activities were analyzed 24 h after transfection (Figure 6B). siRNAs with an asymmetric antisense strand overhang (A3, A3Lm, A3LU and A3Rm) markedly inhibited the luciferase expression from the psiCHECK-RL-Fwd. In contrast, siRNAs with a sense strand overhang (A4, A4Lm, A4Rm and A4RU) predominantly repressed the luciferase expression from the psiCHECK-RL-Rev. Consistent with the results shown in Figure 5, the end stabilities markedly affect the selection of nonoverhang containing strands. For instance, siRNAs A3Lm (o-b) and A3LU (o-b) with a G:G mismatch and G:U wobble pair at the 5′-end of the sense strand respectively, were more effective against the Rev target than siRNA A3 (o-b). This result was observed in the activity of siRNAs with opposite terminal structures [A4Rm (b-o) and A4RU (b-o)]. Importantly, however, we again observed that overhang-containing strands were selected as the guide strand regardless of thermodynamic stabilities. Thus, we conclude that terminal structures of siRNAs duplexes are the predominant determinants of the strand selectivity rather than thermodynamic stabilities at the ends of siRNAs.

Tolerance for deletions or DNA substitutions within the sense strand of asymmetric siRNAs

To further examine the effects of modifications of the sense strand of asymmetric siRNAs on the RNAi activity and antisense strand selection, we made siRNAs variants with deletion of up to 4 nt (del15–18; Figure 7A) from the 3′-end of the sense strand and tested their activities. The sequence

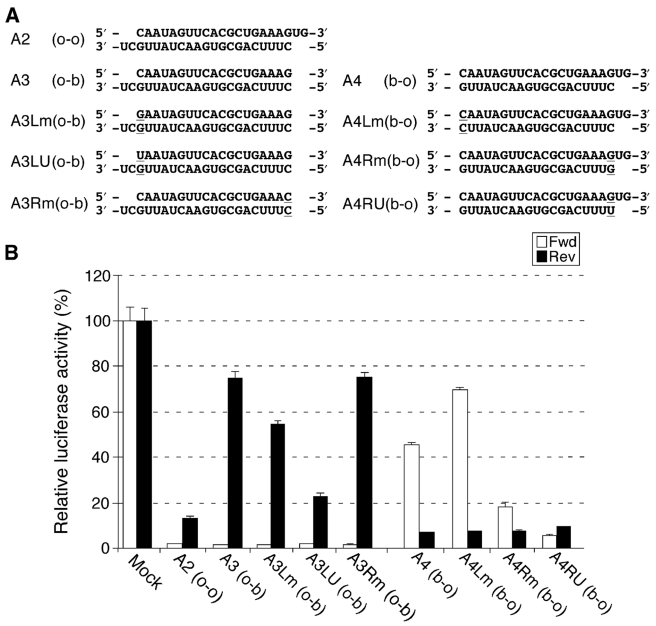

Figure 6. Effects of siRNA terminal structures and stabilities on strand selectivity. (**A**) Sequences of siRNAs with G:U wobble pairs or mismatches. The G:U wobble pairs and mismatches are underlined. (**B**) Inhibition of reporter gene expression by siRNAs with a G:U wobble pair or mismatch. Each of reporter constructs with target sequences in the forward (Fwd) or the reverse (Rev) orientation, and each 1-nM duplex were cotransfected into HeLa S3 cells, and luciferase activities were determined. The activities determined from mock cells were set at 100%. The mean and SD from three replicate assays are presented.

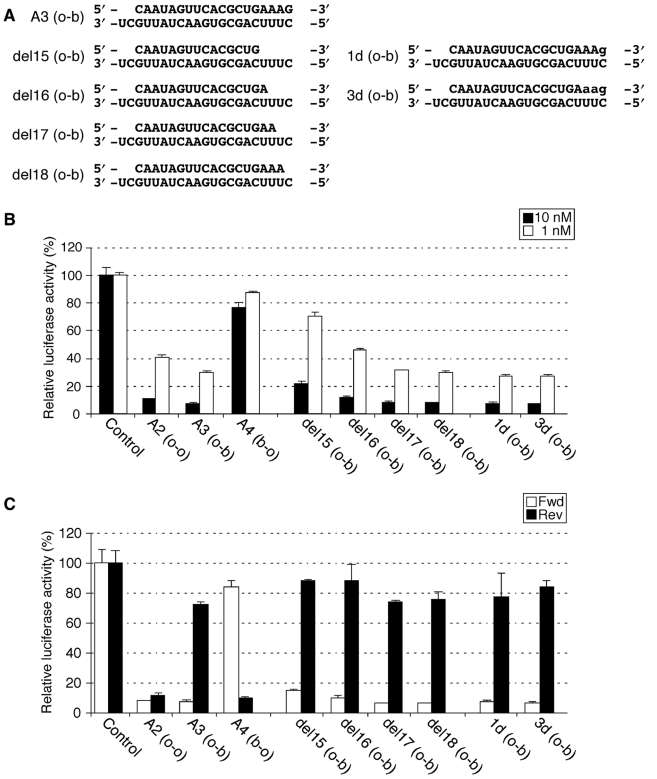

Figure 7. Effects of modifications of siRNAs on RNAi activities and strand selectivity. (**A**) Sequences of siRNA duplexes with modifications. Duplexes contain shortened sense strands (del15–del18) or sense strand DNA-substitutions (1d and 3d), respectively. Substituted DNA sequences are shown in lower case letters. (**B**) Inhibition of the reporter gene expression by modified siRNAs. The *Renilla* luciferase expression plasmid, firefly luciferase expression plasmid and 1- or 10-nM siRNA duplex were cotransfected into HeLa S3 cells, and luciferase activities were determined. The luciferase activities determined from cells transfected with the control siRNA were set at 100%. The mean and SD from three replicate experiments are presented. (**C**) Each reporter construct with a target sequence in the forward (Fwd) or the reverse (Rev) orientation, β-galactosidase expression plasmid and 10-nM modified duplexes were cotransfected into HeLa S3 cells, and luciferase and β-galactosidase activities were determined. The mean and SD are the same as for (B).

of the antisense strand was the same as that of siRNA used in the experiment shown in Figure 1C. Duplexes with 17- and 18-nt sense strand (del17 and del18) showed equal activities relative to the 19mer/21mer duplex (A3) although the 4-nt deletion (del15) reduced the RNAi activity (Figure 7B). Importantly, siRNA variants [del16 (o-b) and del15 (o-b)] showed improved strand selectivity because they were less effective against the Rev target relative to the duplex A3 (o-b) (Figure 7C).

We next examined the effect of DNA substitution at the 3′-end of the sense strand on the RNAi activity as well as strand selectivity. This substitution might increase the stability of siRNAs when used *in vivo*. In addition, Rose *et al.* (23) demonstrated that the DNA substitutions at the blunt end of 27-mer duplexes with an asymmetric antisense overhang improved the guide strand selectivity. Thus, we synthesized modified asymmetric siRNAs with 1-nt or 3-nt DNA residues within the sense strand (1d or 3d; Figure 7A). As shown in Figure 7B, these modified siRNAs were as active as nonmodified siRNA A3 (o-b). When the siRNA 3d was used, the sense strand selectivity was impeded relative to the siRNA A3, indicating that the DNA substitution possibly reduces the sense strand selection (Figure 7C). The preferential antisense strand selection was seen more clearly when siRNA A3-4 (o-b) and A3-5 (o-b) were used (Supplementary Figure 3). Although these siRNAs efficiently inhibited luciferase expression from the Rev target (Figure 5B and Supplementary Figure 3B), due to high-thermodynamic stabilities at 5′-ends of antisense strands relative to 5′-end of the sense strand (Figure 5A), the DNA substitution markedly reduced their activities against the Rev target (Supplementary Figure 3B). These results suggest that partial deletions or DNA substitutions of the sense strand are useful methods to design potent siRNAs with an asymmetric antisense strand overhang.

DISCUSSION

In the present study, we have shown that the siRNA terminal structure is a key determinant of strand selection as well as RNAi activity. siRNAs with an asymmetric antisense strand overhang exhibited improved efficacy relative to siRNAs with symmetric overhangs (Figures 1C and 2). In contrast, siRNAs with an asymmetric sense strand overhang showed significantly reduced activities. We found that these differential activities of siRNAs depend on which strand participates in the RNAi pathway

(Figure 3B and C). Interestingly, siRNA terminal structures predominantly determine the strand selectivity regardless of thermodynamic stabilities at the 5′-ends (Figures 5 and 6). Previous studies by Vermeulen *et al.* (21) and Hohjoh (22) also demonstrated that siRNAs with an asymmetric antisense strand overhang had enhanced efficacy relative to siRNAs with symmetric overhangs. Ohnishi *et al.* (24) further examined the strand selectivity of asymmetric siRNAs termed 'fork-siRNA duplexes'. However, fork-siRNA duplexes contained several mismatches at the 5′-end of antisense strand, and therefore it was not clear whether terminal structures or internal thermodynamic stabilities contribute more to the strand selection of fork-siRNA duplexes. To the best of our knowledge, the present study demonstrates for the first time that the siRNA terminal structure, rather than thermodynamic stability, is the predominant determinant of strand selectivity in mammalian cells.

Our findings provide a useful guideline for the design of more potent siRNAs. The advantage of using siRNAs with an asymmetric antisense strand overhang is that this type of siRNA has a greater activity than conventional siRNAs due to preferential antisense strand selection (Figures 1C, 2 and 3). This feature leads to the reduction of off-target effects attributed to unfavorable sense strand selection. Importantly, a lower stability at the 5′-end of the antisense strand relative to its 3′-end much reduced the sense strand selection as the guide strand (Figures 5 and 6). Therefore, upon selecting target sequences, relative stabilities of both ends of the duplex should be considered in designing asymmetric siRNAs. Alternatively, mismatches at the 5′-end of the antisense strand would help to reduce the sense strand selection. Our results also demonstrated that, for siRNAs with an asymmetric antisense strand overhang, partial deletions or DNA substitutions in their sense strand could improve RNAi potency without significant loss of the RNAi activity (Figure 7B and C and Supplementary figure 3), although a 4-nt deletion compromises the activity (Figure 7B and C). Thus, use of asymmetric siRNAs with these modifications would improve the RNAi effects as well as cost effectiveness relative to conventional siRNAs.

There is great interest in how the mammalian RISC loads one strand of an siRNA. In *Drosophila*, the Dcr-2/R2D2 heterodimer is capable of selecting a guide strand from siRNAs by sensing the thermodynamic stabilities of both ends of siRNAs (20). Recently, it has been shown that this heterodimer is involved in the sorting of siRNAs and siRNA-like miRNA duplexes into Ago2-containing complexes (25,26). In contrast to the *Drosophila* system, it remains unclear how one strand of an siRNA is selected as the guide and promotes RISC maturation in mammals. Our results contribute to the understanding of siRNA strand selection in mammalian cells. We found that the 2-nt overhang structure of siRNA is the predominant determinant of strand selection relative to thermodynamic stabilities of siRNA terminus (Figures 5 and 6). Recent structural studies demonstrated that the PAZ domain of argonaute proteins and of dicer contributes to the recognition of the 2-nt overhang of the dsRNA (27–29). Thus, it is possible that the binding mode of an siRNA to the PAZ domain of Ago2 is important for guide strand selection. In the case of 19mer/21mer asymmetric siRNAs, the Ago2-PAZ preferentially binds to the 3′-end of 21-mer strand and this anchored strand might be recognized as the guide strand. Alternatively, dicer might play a crucial role in the strand selection. Several lines of evidence suggest that dicer is involved in RISC assembly as well as dsRNA processing (23,30–34). Thus, there is the possibility that, as in *Drosophila*, the orientation of dicer on the siRNA duplex determines which strand could be incorporated into the RISC. Dicer may preferentially bind to the asymmetric siRNA terminus with a 2-nt overhang through its PAZ domain and promote the loading of the siRNA duplex into an Ago2-containing complex. Binding of dicer to a 2-nt overhang end of the siRNA may facilitate the presentation of this end to Ago2 and then lead to subsequent selection of the overhang-containing strand as the guide strand. We found that relative stabilities at siRNA termini influence the selection of nonoverhang containing strands (Figures 5 and 6), suggesting that additional factors are likely to play a role in sensing the thermodynamic stabilities of siRNA termini during the RISC assembly. Recent studies suggested that the association of human dicer with its binding partner(s) facilitates siRNA generation as well as the RISC assembly (31,35–38). These partner proteins may direct Ago2 and/or dicer binding to one end of an siRNA to promote siRNA strand selection.

We observed that levels of nonoverhang containing strands in cells were significantly reduced in cells transfected with siRNAs with an asymmetric overhang (Figure 4A). Recent reports demonstrated that Ago2 cleaves the siRNA passenger strand to promote the RISC maturation (39,40). If this is the case, nonoverhang containing strands of an siRNA with an asymmetric overhang may be destroyed by Ago2, as the passenger strand. However, we observed that similar levels of sense and antisense strands accumulated when HeLa S3 cells were transfected with siRNA A4 (b-o) (Figure 4B). Importantly, siRNA A4 (b-o) led to selective reduction of luciferase expression from the reporter plasmid harboring the reverse target sequence (Supplementary Figure 2C and D). These results suggest that selective RNAi activity by siRNAs with an asymmetric 3′-overhang is not always a consequence of selective accumulation of their sense or antisense strand in cells. It is most likely that strand selection occurs after the siRNA duplex is loaded onto Ago2-containing complex (39,40). After this complex distinguishes the guide strand of the siRNA from the passenger strand, the passenger strand could be eliminated from the RISC via Ago2-mediated cleavage (39,40). However, our results suggest that strand selection of siRNAs was not always coupled with destruction of the passenger strand. One explanation for these observations is that strand selection of siRNAs could occur via a cleavage-independent mechanism, as is the case for miRNAs (39,40), depending on siRNA sequence compositions or structures. These siRNA characteristics might also influence the extent of degradation of the passenger strand after release from the RISC.

In summary, our studies demonstrate the potential advantage of using siRNAs with an asymmetric overhang over conventional siRNAs for strand selection and RNAi activity. The siRNA design presented here is simple and cost effective. We have developed a program using our original algorithm, leading to the selection of effective siRNA target sites (41). Combination of this target site selection with the asymmetric siRNA design would contribute to effective RNAi studies *in vitro* as well as *in vivo*. In addition, our findings help in the understanding of strand selection in mammalian cells. Ago2 and dicer play a crucial role in the RNAi pathway. The role of additional factor(s) that interact with dicer and/or Ago2 is presumably of importance in strand selection and RISC assembly. Further functional analyses to clarify how Ago2, dicer and their binding partners are involved in the recognition of siRNAs are required to understand detailed siRNA-loading mechanisms in mammals.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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